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(54) Title: MUTATED HIV TAT

(57) Abstract: The present invention provides a Tat protein wherein all the cysteine residues of the cysteine-rich domain have been replaced with another amino acid, preferably with serine, nucleic acids encoding it, and methods of using it to elicit a humoral and cellular immune responses in a mammal. The Tat protein of the invention is therefore useful, inter alia, for prophylactic and/or therapeutic anti-HIV use as well as raising anti-native Tat antibodies in mammals.



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**MUTATED HIV TAT**  
**BACKGROUND OF THE INVENTION**

**Field of the Invention**

This invention relates to the field of modified HIV Tat nucleic acids and proteins as well as its combination with early HIV proteins and their use in studying the biological mechanism of HIV infection and in vaccine compositions for prophylaxis and treatment of HIV/AIDS.

**Summary of the Related Art**

- 10 HIV Tat protein is an essential viral protein for HIV pathogenesis. It transactivates HIV gene expression by binding to the Trans Activation Response (TAR) element of the HIV RNA Long Terminal Repeat (LTR) region. Tat is released by infected cells in which it is expressed (soluble Tat or sTat) and taken up by other HIV
- 15 infected cells, where it can enter the nucleus and transactivate HIV gene expression. Extracellular Tat induces expression of HIV co-receptors on target cells, thereby further promoting virus spreading. See generally Noonan et al., *Advances in Pharmacology* 48, 229 (2000).
- 20 Tat also plays a role in HIV-induced immunosuppression. *Id.* For example, Cohen et al., *Proc. Natl. Acad. Sci. USA* 96, 10842 (1999), reported that Tat is strongly immunosuppressive, both immediately after immunization of mice with sTat and in seroconverting humans. Tat has also been linked to induction of T-cell anergy and T-cell
- 25 apoptosis, Ross, *Leukemia*, 15, 332 (2001). Furthermore, Tosi et al., *Eur. J. Immunol.* 30, 19 (2000), demonstrated that a modified HIV-1 Tat can act as a immunosuppressor by inhibiting HLA class II expression necessary for triggering both cellular and humoral responses against pathogens.
- 30 The Tat protein is an 86-102 (depending on the HIV strain) amino acid protein encoded by two exons. The first, highly conserved exon contains four functional domains, including the amino-terminal domain (amino acids 1-21), the cysteine-rich domain (amino acids 22-37), the core domain (amino acids 38-48), and the basic domain
- 35 (amino acids 49-57), which is essential for cellular uptake. The cysteine-rich domain is highly conserved and has been reported as being important for the Tat transactivating activity. Individual

mutation in six of the seven cysteines eliminates Tat function. Jeang in *HIV-1 Tat: Structure & Function*, pp. 3-18, Los Alamos National Laboratory (Ed.) Human Retroviruses & AIDS Compendium III. Because of its essential role in HIV expression and propagation, Tat  
5 has been suggested and studied as a possible vaccine. Goldstein, *Nature Medicine*, 1, 960 (1996). Cafaro et al., *Nature Medicine* 5, 643 (1999) reported that vaccination of cynomolgus monkeys with a biologically active HIV-1 Tat protein is safe, elicits a broad (humoral and cellular) specific immune response and reduces  
10 infection of the highly pathogenic simian-human immunodeficiency virus (SHIV)-89.6P to undetectable levels.

For human use suppression or inactivation of Tat activity has been suggested as a route for prophylaxis and/or treatment of HIV infection. E.g., Goldstein, WO 95/31999. Tat protein that has been  
15 modified to reduce or eliminate its transactivating activity while maintaining its immunogenicity has been proposed.

Cohen et al. (*supra*) reported that oxidation of Tat preserves immunogenicity of the protein while inactivating Tat's immunosuppressive effects.

20 Le Buanec and Bizzini., *Biomed & Pharmacother.* 54, 41 (2000), reported on chemical inactivation of Tat, e.g., formaldehyde, glutaraldehyde, and dithionitrobenzoate treatment as well as amidination of lysyl residues, modification of arginyl residues, blockade of sulfhydryl groups by dithionitrobenzoate treatment,  
25 maleimidation, carboxymethylation, and carboxyamidation. They found that such chemical modification resulted in a Tat protein with a partial or complete loss of biological activity but retention of partial to complete antigenicity and immunogenicity in mice compared to native Tat. Zagury et al. (US 6,200,575) also discloses  
30 formaldehyde and glutaraldehyde inactivation of Tat.

Another approach has been modification of the protein by mutation. Caselli et al. investigated two tat genes mutated in the transactivating domain for their ability to elicit an immune response to wild-type Tat in a mouse model. The polypeptides encoded  
35 by the two genes, tat<sub>22</sub> (Cys<sup>22</sup>→Gly) and tat<sub>22/37</sub> (Cys<sup>22</sup>→Gly and Cys<sup>37</sup>→Ser), lack HIV transactivating activity and block wild-type Tat. Caselli et al. injected mice with DNA plasmids containing the

*tat*<sub>22</sub> and *tat*<sub>22/37</sub> genes and tested for humoral and cellular response to wild-type Tat. A humoral response suggestive of a Th1 profile was detected after the third immunization, and mean titers and the number of responder mice increased following three additional boosts  
5 and treatment with bupivacaine (which facilitates DNA uptake in muscle cells and enhances DNA immunization). The response was comparable to DNA immunization with the wild-type *tat* gene.

Caselli et al. also immunized mice with wild-type Tat protein and observed both humoral and cellular responses. Antibody titers were  
10 higher in the Tat-immunized mice compared to the *tat*<sub>22</sub> and *tat*<sub>22/37</sub> immunized mice, although epitope reactivities were more restricted and a Th-2-like response observed. The authors speculated that the differences in DNA and protein immunization response were likely due to protein sensitivities to air, light, and temperature and  
15 differences in presentation of the two to the immune system. Caselli et al. asserted that DNA immunization seemed preferable due to the presence of a cellular response characteristic of a Th1 reaction.

Zagury, (US6,200,575) discloses the use of inactivated Tat and various forms of inactivated Tat as immunogens for prophylactic or  
20 therapeutic immunizations to fight HIV disease.

Tosi et al., (*supra*) reported on a *tat*<sub>22/37</sub> (Cys<sup>22</sup>→Gly and Cys<sup>37</sup>→Ser) and a *tat*<sub>37</sub> (Cys<sup>37</sup>→Ser) mutant, both transfected into T and monocytic cell lines. Both mutants were reported to strongly down-modulate constitutive as well as IFN-γ-inducible HLA class II gene expression  
25 *in vitro*, suggesting that these mutants retain the immunosuppressive function of the native polypeptide.

Goldstein, *Nature Medicine*, *supra*, suggested that a consensus sequences of 21 known HIV-1 Tat proteins could be used as the immunogen in a vaccine and further suggested Cys → Ser substitutions  
30 could be made at positions 22, 25, 27, and/or 37 to block transactivation without affecting the immunogenic domains.

Goldstein WO 95/31999 suggested inactivation of Tat by deletion at the amino or carboxy terminus or deletion or replacement of native cysteine residues to interfere with naturally-occurring disulfide  
35 bonds.

Loret (WO 00/61067) discloses Tat protein mutated in the cysteine rich region. Most particularly, Loret specifically considers Tat OYI, which corresponds to a Tat protein having a natural Cys<sup>22</sup>→Ser mutation.

5 Furthermore, Osterhaus et al. has demonstrated the presence of Tat and Rev-specific CTL in seropositive long term non progressors whereas these CTLs were not found in patients progressing to disease. In addition immunization of macaques with a combination of vectors expressing the SIV tat and rev genes protected the animals  
10 against pathogenic SIV challenge. Vaccine 17, 27-31, 1999; U.S. 6024965.

A recent study by Addo et al. (*Proc. Natl. Acad. Sci. USA* vol. 98, 1781-1786) demonstrated that controllers (HIV-1 infected individuals capable of controlling viremia without medication) had CTLs  
15 targeting more epitopes in Tat relative to individuals on drug treatment. Furthermore, the anti-Tat CTL responses were also of higher magnitude in controllers.

More recently, Allen et al. demonstrated that Tat-specific CTLs are involved in controlling wild-type virus replication during SIV  
20 infection of rhesus macaques. *Nature* 407, Sept 2000, 386-390.

Despite the tremendous effort that has been dedicated to the study of HIV, Tat, early proteins and their role in AIDS, all of the molecular biological mechanisms of HIV in general and Tat in particular are not completely known or understood. A composition and  
25 method for HIV/AIDS prophylaxis and treatment has also remained elusive. Accordingly, there still remains a need for an HIV/AIDS vaccine as well as useful research tools to study HIV infection.

All patents and other publications recited herein are hereby incorporated by reference in there entirety.

### 30 SUMMARY OF THE INVENTION

The present invention is based on the discovery that modification of HIV Tat protein in the cysteine rich domain by replacing all the cysteine residues with other amino acids, preferably serine, results in a modified Tat protein that retains its immunogenicity, is unable  
35 to transactivate HIV expression, is not immunosuppressive, and is able to induce neutralizing antibodies. The present invention

comprises also the simultaneous use of tat, rev and nef genes to elicit broad HIV specific T cell responses (including CD4 and CD8 as well as innate immunity). This combination of features makes the modified Tat protein of the invention as well as its combination  
5 with early proteins useful both as a vaccine as well as a research tool to study the molecular and systemic mechanisms involved in HIV infection.

The present invention thus provides a Tat protein comprising a mutated cysteine-rich domain wherein all the cysteine residues of  
10 the cysteine-rich domain have been replaced independently with another amino acid.

According to a specific embodiment each cysteine residue of the cysteine-rich domain is a conservative substitution and is preferably a serine.

15 In another aspect, the invention relates to a nucleic acid encoding the Tat protein as defined above as well as an expression vector comprising said nucleic acid. In alternative embodiments, the said vector further comprises a DNA sequence encoding Nef and Rev proteins. According to a preferred embodiment, the DNA sequence  
20 encoding the Rev protein is inserted anywhere into the nef DNA sequence encoding amino acids 150-179 of the Nef protein.

In another aspect, the invention provides a composition comprising the above-defined Tat protein or expression vector in combination with a carrier and optionally an adjuvant, especially at least one  
25 Th1 adjuvant. Such composition is use for *in vitro* and *in vivo* administration both as an anti-HIV vaccine as well as for the purpose of studying HIV infection.

The present invention also relates to a method of eliciting a humoral and cellular immune response in a mammal comprising  
30 administering the above-defined composition to the mammal. According to a specific embodiment, the composition comprising the Tat protein of the invention is administered simultaneously or sequentially with the composition comprising the expression vector of the invention.

The foregoing merely summarizes certain aspects of the invention and  
35 is not intended, nor should it be constructed, as limiting the invention in any manner. Additional details of the invention are provided below. All patents, patent applications, and other

publications recited in this specification are hereby incorporated by reference in their entirety.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 displays the results of immunosuppressive activity of various Tat measured *in vitro* by a lymphoproliferation assay.

Figure 2 displays anti-Tat<sub>IIIB</sub> IgG ELISA titers of guinea pigs immunized with various Tat proteins.

Figure 3 displays the results of the transactivation assay.

Figure 4 gives the plasmid map of pET8cTat.

### 10 DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the invention thus provides a Tat protein comprising a mutated cysteine-rich domain wherein each cysteine residue of the cysteine-rich domain has been replaced with another amino acid, preferably a conservative amino acid, most preferably a serine.

As used herein a "Tat protein" means any naturally occurring Tat protein obtained from any HIV-1, HIV-2 or SIV strain, including laboratory and primary isolates. The Tat protein is obtained preferably from a HIV-1 strain and more particularly from a HIV-1 IIIB strain. Two kinds of Tat proteins have been disclosed in the literature *i.e.*, Tat proteins having a short sequence of 86 amino acids and Tat proteins having a longer sequence of up to 99 to 102 amino acids. This difference in size has been attributed to the variable length of the second exon encoding the protein. These two types of proteins fall under the scope of the invention. The amino acid sequences of a large number of Tat proteins are known and available, *e.g.*, "Human Retroviruses and AIDS 1999: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences," Kuiken *et al.*, Eds., Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM, and <http://hiv-web.lanl.gov/>, and any of these can be used in the present invention. The Tat protein is composed of various conserved functional domains, and comprises particularly a highly conserved cysteine-rich domain. This definition also encompasses the said Tat proteins in which mutations have been introduced with the proviso that the said proteins contain a mutated cysteine-rich domain as defined below and remain devoid of

any transactivating and immunosuppressive activity and further remain capable of inducing neutralizing antibodies and a cellular immune response. The Tat protein of the invention is preferably Tat IIIB and corresponds most preferably to SEQ ID No 1.

5 As used herein, the "mutated cysteine-rich domain" is the sequence corresponding to amino acids 22 to 37 of the Tat protein wherein each cysteine residue at positions 22, 25, 27, 30, 31, 34 and 37 have been independently replaced with another amino acid, corresponding preferably to a conservative substitution and most  
10 preferably to a serine residue. This definition intends also to include cysteine-rich domains in which in addition to the above-mentioned mutations, additional conservative substitution(s) have been introduced in positions different from positions 22, 25, 27, 30, 31, 34 and 37. Taking as a reference the cysteine-rich domain of  
15 Tat IIIB, this definition includes all cysteine-domains having a similarity with IIIB cysteine-rich domain of at least 50%, preferably of at least 75%, most preferably of 100%.

A "conservative amino acid substitution" is a substitution of a native amino acid residue with a nonnative residue such that there  
20 is little or no effect on the polarity or charge of the amino acid residue at that position. A "conservative amino acid substitution" also encompasses non-naturally occurring amino acid residues that are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics,  
25 and other reversed or inverted forms of amino acid moieties.

Naturally occurring residues may be divided into classes based on common side chain properties:

- 1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
- 2) neutral hydrophilic: Cys, Ser, Thr;
- 30 3) acidic: Asp, Glu;
- 4) basic: Asn, Gln, His, Lys, Arg;
- 5) residues that influence chain orientation: Gly, Pro; and
- 6) aromatic: Trp, Tyr, Phe.

For example, non-conservative substitutions may involve the exchange  
35 of a member of one of these classes for a member from another class. In making such changes, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on



the basis of its hydrophobicity and charge characteristics. The hydrophobic indices are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydrophobic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte et al., 1982, *J. Mol. Biol.* 157:105-31). It is known that certain amino acids may be substituted for other amino acids having a similar hydrophobic index or score and still retain a similar biological activity. In making changes based upon the hydrophobic index, the substitution of amino acids whose hydrophobic indices are within  $\pm 2$  is preferred, those which are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the biologically functionally equivalent protein or peptide thereby created is intended for use in immunological embodiments, as in the present case. The greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e., with a biological property of the protein.

The following hydrophilicity values have been assigned to these amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0  $\pm$  1); glutamate (+3.0  $\pm$  1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5  $\pm$  1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4). In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within  $\pm 2$  is preferred, those which are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred. One may also identify epitopes from primary amino acid sequences on the basis of

hydrophilicity. These regions are also referred to as "epitopic core regions."

Desired amino acid substitutions (whether conservative or non-conservative) can be determined by those skilled in the art at the  
5 time such substitutions are desired.

The term "similarity" refers to a measure of relatedness that includes both identical matches and conservative substitution matches between two sequences as determined by a particular mathematical model or computer program (i.e., "algorithms") by  
10 inserting gaps, if required, in one or both sequences. A suitable programs available for public use is FASTA. If two polypeptide sequences have 10 of 20 identical amino acids, for example, and the remainder are all non-conservative substitutions, then the percent identity and similarity would both be 50%. If in the same example  
15 there are five positions in which there are conservative substitutions (in addition to the 10 identical residues), then the percent identity remains 50%, but the percent similarity would be 75% (15/20).

In a preferred embodiment of the Tat protein of the invention, amino  
20 acid residues at positions 22, 25, 27, 30, 31, 34, and 37 are serine residues (herein called Tat7C/S). According to a preferred embodiment, Tat7C/S corresponds to Tat IIIB 7C/S.

In another embodiment, the Tat protein of the present invention is further modified by chemically methods such as those disclosed in  
25 U.S. 6,200,575.

Amino acid numbering used herein is based on the sequence of the HIV-1 viral strain III B. The Tat protein of this strain is (SEQ ID NO 1):

MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVS  
30 LSKQPTSQSRGDPTGPKE

Whenever a number of an amino acid residue or sequence is used in reference to a sequence other than from the IIIB strain, that number refers to the residue or sequence that corresponds to the numbered residue or sequence in the IIIB Tat.

35 The Tat proteins of the invention can be made routinely using methods known in the art. The proteins can be synthesized or, preferably, expressed from a vector in a suitable expression system.

Vectors and expression of the encoded Tat protein of the invention is described fully below. When the Tat protein is produced by chemical synthesis, it is possible either to produce it in the form of one sequence or in the form of several sequences that are subsequently linked together in the correct order. The chemical synthesis may be carried out on solid phase or in solution, these two technologies being well known to the person skilled in the art and are described for example by the following authors: Atherton and Shepart "solid phase peptide synthesis" (IRL press Oxford, 1989);  
10 Houbenweyl "Method der organischen chemie" editor E. Wunsch vol 15-I and II, Stuttgart 1974; Dawson PE and al "Synthesis of proteins by native chemical ligation" Science, 1994, 266 (5186): 776-9; Kochendoerfer GG and al "Chemical protein synthesis" Curr. Opin. Chem. Biol., 1999, 3(6):665-71; and Dawson PE and al "Synthesis of  
15 native proteins by chemical ligation" Annu. Rev. Biochem. 2000, 69: 923-60. The protein thus produced may be easily isolated and purified by methods well known in the art.

The protein of the invention may also be produced by recombinant technologies well known in the art. These methods are described in  
20 details in the last edition of "Molecular Cloning: A Molecular Manual" by Sambrook et al., Cold Spring Harbor, *supra*. In such a case, the DNA sequence encoding the Tat protein of the invention is first produced by directed mutagenesis starting from the wild-type DNA sequence encoding Tat. Such a step may be carried out by PCR  
25 using primers containing the DNA sequence encoding the mutation(s) to be introduced. The mutated DNA sequence is then inserted into an appropriate expression vector. The thus obtained recombinant vector is then used to transform appropriate host cells to express the mutated Tat protein. The protein thus produced is isolated and  
30 purified using methods well known in the art. A process of expression and purification of the protein according to the invention is described in details in the attached examples. The process of the invention leads advantageously to a highly purified monomeric Tat protein which does not form any aggregates.

35 Concerning the "expression vector," any expression vector classically used for the expression of recombinant proteins can be used to produce the Tat protein of the invention. "Expression

vectors" thus encompass live expression vectors such as viruses and bacteria as well as plasmids. Vectors in which the expression of the Tat DNA sequence is controlled by an inducible or non-inducible strong promoter are advantageously used. Expression vectors may include a selection marker such as, for example, an antibiotic resistance gene (such as Kanamycin) or dihydrofolate reductase gene. Non-limitative examples of expression vectors that can be used in the process of production of the Tat protein of the invention include: pET28 (Novagen), pBAD (Invitrogen) plasmids; viral vectors such as baculovirus, adenovirus, adeno-associated virus (AAV), poxvirus (including avian pox, fowl pox, and preferably the attenuated vaccinia vector NYVAC (U.S. 5,364,773) or MVA (modified vaccinia virus Ankara, Swiss Patent No.: 568,392 and U.S. 5,185,146), and the attenuated canarypox vector ALVAC (U.S. 5,756,103; U.S. 5,990,091), poliovirus, alphavirus, VSV, herpes and retroviral vectors, as well as bacterial vectors such as salmonella, shigella and BCG.

To obtain the expression of the Tat protein, any host cell classically used in association with the above-mentioned vectors can be used in the present invention. Non limitative examples of such host cells include cells from *E. coli* such as BL21( $\lambda$ DE3), HB101, Topp 10, CAG 1139, cells from bacillus, and eukaryotic cells such as Vero, BHK, MRC5, MDCK, PERC-6, and CHO cells.

The expression system preferably used in the present invention corresponds to the pM1815/*E. coli* cells.

In another aspect, the invention relates to the nucleic acid sequences encoding the above-defined Tat protein of the invention.

The nucleotide sequences of a large number of *tat* genes are known and available, e.g., on the web site: <http://hiv-web.lanl.gov/>.

Nucleic acid numbering used herein is based on the following *tat* DNA sequence from HIV-1 viral strain III B (Seq. ID. No.: 2):

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atggagccag tagatcctag actagagccc tggaagcatc caggaagtca gcctaaaact
gcttgtagca attgctattg taaaaagtgt tgctttcatt gccaaagtttg tttcataaca
aaagccttag gcatctccta tggcaggaag aagcggagac agcgacgaag acctcctcaa
ggcagtcaga ctcatcaagt ttctctatca aagcaaccca cctcccaatc ccgagggggac
ccgacaggcc cgaaggaata gaagaagaag gtggagagag agacagagac agatccattc
gattagttaa

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The bold/underline codon indicates a stop codon at position 259 (with an X in the corresponding position in the amino acid sequence) in the IIIB Tat, which, accordingly is 86 amino acids long. A number of naturally occurring Tat sequences have a Glu or Ser codon in place of this stop codon and have an additional 14 or more amino acid residues at the carboxy terminal end.

Whenever a number of a nucleic acid residue is used in reference to a sequence other from the IIIB strain, that number refers to the residue that corresponds to the numbered residue in the SEQ ID No 2 sequence.

When a first nucleic or amino acid residue or sequence within a first polynucleotide or polypeptide (respectively) aligns with a second nucleic or amino acid residue or sequence within a second polynucleotide or polypeptide (respectively) when the two polynucleotides or polypeptides are brought into alignment using any art recognized alignment algorithm, e.g., SIM (Xiaoquin et al., *Advances in Applied Mathematics* 12, 337 (1991)), the first nucleic or amino acid residue or sequence within a first polynucleotide or polypeptide (respectively) are said to "correspond" one to the other.

The codons of the nucleic acids of the invention can be advantageously optimized to improve the expression level, the selection of the optimized codons depending on the selected host cells.

In a third aspect, the invention comprises an expression vector encoding the nucleic acid of the second aspect of the invention. Expression vectors into which the nucleic acids of the second aspect of the invention may be inserted are well known in the art and can be routinely selected by those of ordinary skill in the art based primarily on the host system into which the vector is to be inserted. Methods for inserting the nucleic acids of the second aspect of the invention into vectors are well known and routinely applied. E.g., Sambrook et al., "Molecular Cloning: A Laboratory Manual" vols. 1-3 (3<sup>rd</sup> Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York 2001).

Expression vectors that can be employed in this aspect of the invention have been described in detail in the section regarding the

process of production of the Tat protein. The expression vectors of the present invention can be used either for the production of the Tat protein or directly as an active vaccine component of a composition of the invention. When the expression vector is used as  
5 a vaccine component, the expression vector to be used does not comprise any selection marker and corresponds to a viral vector such as adenovirus, poxvirus (including fowl pox, avian pox, and preferably the attenuated vaccinia vector NYVAC (US 5,364,773) or MVA (modified vaccinia virus Ankara, Swiss Patent No.: 568,392 and  
10 US 5,185,146), and the attenuated canarypox vector ALVAC (US 5,756,103; US 5990091), poliovirus, alphavirus, VSV, herpes retroviral vector, or a bacterial vector such as salmonella, shigella or BCG, or a plasmid DNA vectors including layer DNA vectors.

15 In one embodiment of this aspect of the invention, the nucleic acid encoding the modified Tat polypeptide of the invention is the only HIV/SIV immunogen encoded by the vector.

In a preferred embodiment, the vector according to this aspect of the invention further comprises nucleic acid sequences encoding the  
20 Rev and Nef HIV-1 proteins. Numerous wild-type rev and nef nucleic acid sequences are known. Figures 9-11 and 15-17 display many of them, and we contemplate that any of those displayed as well as consensus sequences of any two or more of these sequences can be used in the invention. In this embodiment, the vector of the  
25 invention comprises a nucleic acid sequence according to the second aspect of the invention and both a rev and nef sequence, and the vector express the mutated Tat protein of the invention and Rev and Nef proteins in the intended host. Preferably, in this embodiment the rev DNA sequence is inserted into the nef DNA sequence.  
30 Preferably, the rev DNA sequence is inserted anywhere into the region coding for amino acids 150-179 of Nef, thus producing an inactivated Nef protein without altering the CTL epitopes of the protein.

As used here "Nef and Rev proteins" means any naturally occurring  
35 Rev and Nef proteins obtained from any HIV-1, HIV-2 or SIV strain including laboratory and primary isolates. The Rev and Nef proteins are obtained preferably from a HIV-1 strain. The DNA sequences

inserted in the expression vector of the invention comprises preferably the consensus sequences of the DNA sequences encoding Rev and Nef or DNA fragments thereof coding for CTL epitopes. The amino acid and nucleotide sequences of Rev and Nef as well as the CTL epitopes thereof so far identified can be download from the web site: <http://hiv-web.lanl.gov/>.

In a preferred embodiment, the expression vector expresses the DNA sequence encoding the Tat protein under one promoter and the DNA sequences encoding Rev and Nef under another promoter. This construct advantageously produces an immunologically active Tat protein capable of being secreted by mammalian cells, taken up by mammalian cells, is presented as antigen and is recognized by immune cells and/or specific antibody.

The modified Tat proteins of the invention have several uses. They can be used alone or as a component of a prophylactic or therapeutic vaccine, where its inability to transactivate HIV gene expression and induce immunosuppression in a host while retaining its immunogenicity and capacity to produce neutralizing antibodies and cellular immune response make it both safe and effective for HIV infection prophylaxis and treatment.

The transactivating and immunosuppressive activities of the Tat protein can be easily determined by the CAT assay and the immunosuppression assay, respectively, as described in the attached examples. The induction of neutralizing antibodies can be easily demonstrated by the neutralization assay as described in the attached examples.

The present invention thus also provides compositions, especially vaccines, comprising a Tat protein and/or an expression vector as defined above in combination with a suitable carrier.

The nature of the carrier will vary depending on the intended application. For example, for *in vitro* assays, the carrier can be a simple buffer solution. For prophylactic or therapeutic purposes, the carrier can be any pharmaceutically acceptable carrier, many of which are known in the art. A pharmaceutically acceptable carrier will also be desirable for uses *in vivo* other than treatment or prophylaxis, e.g., raising anti-Tat antibodies for use in assays or treatment.

Methods of making pharmaceutical compositions are well known and can be routinely used to make pharmaceutical compositions according to the fourth and fifth aspects of the invention. *E.g.*, "Remington: The Science and Practice of Pharmacy," by Alfonso R. Gennaro (20th  
5 edition, Lippincott, Williams & Wilkins, Philadelphia, PA, 2000).

According to one embodiment, the composition comprises Tat7C/S in combination with a pharmaceutically acceptable carrier. Such a composition may be stored in lyophilized form and reconstituted in an injectable solution before injection.

10 The composition of the invention may include one adjuvant such as a Th1 adjuvant (*e.g.*, CpG sequences or MPL and MPL analogs), or a Th2 adjuvant (*e.g.*, alum, emulsions, minerals) or a combination adjuvant including at least one Th1 adjuvant.

As part of a vaccine the Tat protein of the invention can also be  
15 used in a lipidated form comprising a lipidic part covalently linked to the Tat protein. Lipidic parts appropriate to form such lipidated Tat as well as a process of preparation of the same can be found *e.g.*, in US5993823. The lipidated Tat protein comprises preferably a N- $\epsilon$ -lysylpalmytoyl residue linked at the COOH terminal function of  
20 the Tat protein.

As part of a vaccine, it can be the sole immunogen or one of several. The Tat protein can be used as the sole immunogen of therapeutic anti-HIV vaccine. Preferably the protein of the invention is used in combination with an expression vector  
25 expressing the Tat protein of the invention in combination with Rev and Nef in order to produce an anti-HIV prophylactic or therapeutic vaccine. Tat, Rev and Nef are HIV proteins expressed early during the infection cycle, before production of infectious virions. These proteins are processed and CTL epitopes are expressed in the context  
30 of HLA class I antigen on the surface of HIV-infected cells. The advantage of immunizing humans against these three proteins altogether is to induce cytotoxic T cells capable of killing HIV infected cells before virions can be produced thus eradicating infected cells and preventing HIV replication and spreading. Also,  
35 one of the functions of the viral Nef protein is to down-regulate MHC class-I molecule expression on the cell surface and thereby confer resistance to immune recognition by CD8 cells. Once the



structural proteins are made, there is presumably sufficient Nef already present to confer resistance to cytotoxic T cells. The Nef used in this embodiment as a vaccine, therefore, should be devoid of this activity.

5 Furthermore, the protein and the expression vector of the invention may also be combined with other subunits HIV immunogens or vectors encoding the same such as Env, Gag, Pol, Vpr, Vpu and Vif. Advantageously, the Tat protein of the invention may be combined with the ALVAC constructions, especially ALVAC 1452 and 1433 as  
10 disclosed in US 5990091.

Such vaccines can be prepared by standard methods well known to those of ordinary skill in the art with standard vaccine pharmaceutical carriers and, preferably, with an adjuvant.

In a sixth aspect, the invention provides a method of eliciting a  
15 humoral and cellular immune response in a mammal, comprising administering to a subject (preferably human) one or more compositions according to the fourth and/or fifth aspect of the invention to elicit humoral and cellular immune responses.

"Cellular immune response" means induction of a specific CD4 T cell  
20 response optionally in association with a specific CD8 T cell response and an innate immune response.

CD4 T cell responses can be monitored upon in vitro recall of peripheral or splenic mononuclear cells with the antigen used to immunized animals. Lymphoproliferative responses as well as cytokine  
25 inductions (Th1/Th2 balance) can be measured (for a review see MK Jenkins, Annu rev Immunol. 2001, 19, 23-45).

CD8 T cell responses can be evaluated (ex vivo or upon re-stimulation of mononuclear cells) either using 1) a standard Chromium release assay which directly measures antigen specific  
30 lytic activity (P. Brossard et al., Blood, 90, 1594-1599) or using IFN $\gamma$ ELISPOT or ICC (intracellular cytokine) assays that both measure the ability of CD8 cells to be stimulated by a 9mer peptide specific for the antigen versus an irrelevant 9mer peptide (Carvalho LH et al., J. Immunol. Methods 2001; 252, 207-18) for IFN $\gamma$ ELISPOT and (C  
35 King et al., Nature Medicine, 7, 206-212) for ICC.

Innate immune responses can be monitored by measuring the levels of pro-inflammatory (IL-6, TNF $\alpha$ ) and/or anti-viral (type I interferons)

cytokines in the serum of immunized animals or upon in vitro antigen specific re-stimulations. The early stimulation of innate immunity can also be evaluated by assessing the ex vivo activation status of antigen presenting cells (monocytes, dendritic cells) and NK cells  
5 that are derived from recently immunized animals (L Krishnan et al., J. Immunol. 2001, 166, 1885-1893).

According to a preferred embodiment, a composition of the invention comprising a Tat protein is administered simultaneously or sequentially, preferably co-administered, with a composition  
10 comprising an expression vector of the invention, preferably an expression vector expressing in addition to the Tat protein of the invention the Rev and Nef proteins.

Suitable amounts of protein for vaccine and other in vivo applications are 10 to 500, preferably 20 to 200 µg per dose.  
15 Suitable amounts of viral expression vectors are in the range of  $10^4$  to  $10^{11}$  pfu, and suitable amounts of plasmid expression vectors is 0.1 to 5 mg per dose.

Administration according to this aspect of the invention can thus be of a protein composition according to the fourth aspect of the  
20 invention, a vector according to the fifth aspect of the invention, or both, either simultaneously or sequentially. Furthermore, administration may comprise compositions of more than one protein, or expression vector. For example, one or a combination of composition comprising DNA plasmid plus a viral vector or two  
25 vectors expressing the same genes can be administered (e.g., DNA plasmid-tat/rev/nef + Pox-tat/rev/nef or Alphavirus-tat/rev/nef + Pox-tat/rev/nef or). In an alternative embodiment, administration according to this aspect of the invention can be a combination of vectors carrying different genes (e.g., vector-tat/rev/nef + vector-  
30 gag/pol/env). In each instance, the number of injections is preferably 2 to 5 for each vector. Furthermore, the number of injections is also preferably of 2 to 5 for the composition comprising the Tat protein of the invention.

Administration of the composition of the invention can be carried  
35 out by intradermal, mucosal route or preferably by intramuscular injection.

The method of this aspect of the invention is useful for prophylactic and therapeutic treatment of HIV infection. The method is also useful to raise anti-Tat antibodies in a healthy mammal or a mammal infected with HIV without further harming the mammal. The antibodies thereby raised can be harvested and used for treatment, for assays, and for the study of the molecular and systemic effects of anti-Tat antibodies on HIV infection.

The Tat protein of the invention can be used to raise anti-wild-type Tat antibodies in mammalian systems susceptible to AIDS without otherwise compromising the health of the mammal. Such antibodies can be used to further study the immune response to HIV, in HIV assays, as well as to treat HIV infection.

The Tat protein of the invention can be used to produced monoclonal antibodies by methods well known in the art directed against specific epitopes of the protein. These antibodies could be used for passive Immunotherapy of HIV-infected individual in combination with chemotherapy and or therapeutic vaccination.

The said monoclonal antibodies can be used in ELISA assays. They are particularly useful as a prognostic tool to detect Tat antigenemia in course of HIV-infection inasmuch as the serum concentration of Tat is correlated with the number of HIV-infected cells.

Furthermore, the Tat protein of the invention can be used in ELISA assays to detect anti-Tat antibodies present in the serum of treated or non treated HIV-infected patients since high level of anti-Tat antibodies correlates with non progression to disease as demonstrated by Zagury et al. (*J. of Human Virology*, 1998, 1, 282-292). In such a case the protein of the invention is coated on an ELISA plate, contacted with serial dilutions of the patient serum to be tested, and then contacted with a enzyme-linked anti-human antibody. The anti-human antibody/anti-Tat antibody complex thus formed is then detected by colorimetric detection. The Tat protein of the invention can be advantageously us as a negative control in any assay aiming to evaluate the transactivating and/or immunosuppressive activity of a Tat protein.

The tat/rev/nef expression vector of the invention can be used in ELISPOT assays to measure cellular responses in seropositive individuals as well as vaccinated individuals immunized with a

different vector. Indeed, Tat and Rev responses have been shown to correlate with long-term non-progression. Carel A. Van Baalen *et al.*, *J. of General. Virology* **78**, 1913-1918 (1997).

- Another use of the mutated Tat protein of the invention is as a research tool to study the immune response to HIV Tat during HIV infection. The mutated Tat protein of the invention enables scientists to observe the immune response to Tat in a model *in vivo* system without the presence of the complicating molecular processes of HIV gene expression and Tat induced immunosuppression.
- 10 The following examples further illustrate the invention and are not intended, nor should they be construed as limiting the invention in any manner. Those skilled in the art will appreciate that variations of the Examples provided below can be made in accordance with the teachings herein and knowledge common to those skilled in the art
- 15 without varying from the scope or spirit of the present invention.

## EXAMPLES

### Example 1

#### Construction of plasmid pET8cTat7C/S

The construction of this clone involved two steps:

- 20 I the directed mutagenesis of the WT-tat gene to obtain the triple-mutant clone : Cys 30, Cys 31, Cys 34 → Ser 30, Ser 31, Ser 34.
- II the directed mutagenesis of the tat-triple-mutant gene to obtain the pET8cTat7C/S plasmid.

#### 25 Mutagenesis and cloning of the triple mutant of Tat

We used the recombinant PCR technique to mutate the WT-tat IIIB gene. The template was the clone pET8cTat (containing Seq. ID. No.: 2). The map of this plasmid is given in Figure 4 and its entire DNA sequence is given in SEQ ID No 10. The recombinant PCR technique

30 requires two PCR steps.

In the first step, two PCR reactions lead to the amplification and the mutagenesis of two overlapping fragments: the "5' fragment" and the "3' fragment" of the *tat* gene.

In the second round, the two overlapping fragments are mixed

35 together along with 5' and 3' primers to amplify the whole mutated *tat* gene. In the strategy outlined below, nucleotide positions in

the PCR primers corresponding to targeted alterations are underlined.

**The protocol used was:**

1. First round of PCR : amplification and mutagenesis of the 5' fragment using the following primers : PBAMU(5' - CGCGGATCCATGGAGCCAGTAGATCCTA-3') (SEQ ID No 3) and R8 (5' GTTATGAAACAACTTGGGAATGAAAGGAAGACTTT-3') (SEQ ID No 4) and amplification and mutagenesis of the 3' fragment using the following primers: PHINDR (5' CCCCAAGCTTCACTAATCGAATGGATCT-3') (SEQ ID No 5) and U8(5'-AAAGTCTTCCTTTTCATTCCCAAGTTTGTTCATAAC-3') (SEQ ID No 6)
2. Purification of these two PCR products using a preparative 2.5 % agarose gel and a Qiagen gel extraction Kit (Qiagen, Valencia, CA)
3. Second round of PCR : amplification of the whole mutated gene using both fragments from the first round of PCR and the two primers : PBAMU (SEQ ID No 3) and PHINDR (SEQ ID No5)
4. Purification of the 327 bp triple-mutated Tat-gene using a preparative 2.5 % agarose gel and a Qiagen gel extraction Kit (Qiagen, Valencia)
5. Digestion of these DNA fragment by Hind III and Bam HI and purification of the fragment using a Qiagen PCR Extraction Kit.
6. Ligation of the digested fragment into pET8c vector previously digested with Bam HI and Hind III, transformation of XL 10 competent bacteria (Invitrogen, Carlsbad) with the ligation mix and mini-preparation of plasmids from cultures grown from the transformants using Qiagen Mini-Prep kit (Qiagen, Valencia)
7. Restriction analysis of the clones obtained and DNA sequencing.

Mutagenesis and cloning of the 7-serine mutant of tat

- 30 The recombinant PCR technique was used with the triple mutant clone (obtained in the previous step) as template.

However, we needed 3 PCR steps to successfully amplify and mutate the whole gene. We were unsuccessful initially in trying to perform the recombinant PCR step with the initial length of overlap, so we extended the 5' PCR products to increase the length of overlap between the two PCR products to be recombined in the final step. By

combining the extended mutated 5' fragment with the 3' fragment in a third round of PCR using the 5' and 3' terminal primers, we were able to generate the full length 7C/S fragment.

**The protocol used was:**

- 5 1. First round of PCR : amplification and mutagenesis of the 5' fragment using the following primers PBAMU (5' CGCGGATCCATGGAGCCAGTAGATCCTA-3') (SEQ ID No 3) and R9 (5' AAAGGAAGACTTTTTAGGAATGGTAGAAGCAGTTTT-3') (SEQ ID No 7) and amplification and mutagenesis of the 3' fragment using the  
10 following primers : PHINDR (5' CCCCAAG-CTTCACTAATCGAATGGATCT-3') (SEQ ID No 5) and U10 (5'- TAAAAAGTCTTCCTTTCATTCCCAAGTTT-CTTTCATAACAAA-3') (SEQ ID No 8)
2. Purification of these two PCR products using a preparative 2.5 % agarose gel and a Qiagen gel extraction Kit (Qiagen, Valencia)
- 15 3. These two fragments failed to generate the full length Tat fragment in a secondary PCR reaction. Therefore we extended the 5' fragment to increase the region of overlap. This step # 3 enabled the extension of the 5' fragment by PCR using the primers PBAMU(5'-CGCGGATCCATGGAGCCAGTAGATCCTA-3') (SEQ ID No 3) and  
20 R11 (5'- GAAAGAACTTG-GGAATGAAAGGAAGACTTTTTAGGAATAGG-3') (SEQ ID No 9)
4. Purification of this extended fragment using a preparative 2.5 % agarose gel and a Qiagen gel extraction Kit (Qiagen, Valencia)
5. Third round of PCR amplification of the whole mutated gene using  
25 both fragments from the first round (fragments 3', step # 1) and second round of PCR (extended fragment 5', step #3) and the two primers : PBAMU (SEQ ID No 3) and PHINDR (SEQ ID No 5)
6. Purification of the 327 bp 7-ser-mutant-Tat-gene using a preparative 2.5 % agarose gel and a Qiagen gel extraction Kit  
30 (Qiagen, Valencia)
7. Digestion of these DNA fragment by Hind III and Bam HI and purification of the fragment using a Qiagen PCR Extraction Kit.
8. Ligation of the digested fragment into pET8c previously digested with Bam HI and Hind III, transformation of XL 10 competent  
35 bacteria (Invitrogen, Carlsbad) with the ligation mix and mini-preparation of plasmids using Qiagen Mini-Prep kit (Qiagen, Valencia)

9. Restriction analysis of the clones obtained and DNA sequencing for confirmation of the desired construct.

### Example 2

Construction of plasmid pM1815

- 5 The Tat7C/S gene was inserted in the plasmid pET8cTat7C/S of example 1 between the BamHI and HindIII sites. Since the ATG start site was immediately downstream of the Bam HI site (ggatccATGg) in the pET8cTat7C/S, this created an NcoI site (CCATGG) at the translation initiation codon. This NcoI site permitted direct  
10 insertion without modification of the reading frame in the pM1800 plasmid. This gene was therefore reinserted in this plasmid between the 5'NcoI and 3'HindIII sites.

- The plasmid pM1800 is constructed starting from pET28 (Novagen). pET28c was amplified by PCR using two primers flanking either side  
15 of the region corresponding to the origin fl. The product thus amplified corresponds comprises the whole sequence of the vector with the exception of the region comprising origin fl. The two restriction sites Asc I and Pac I are introduced via the two primers used in the PCR reaction. In parallel the cer fragment is amplified  
20 using two primers which lead to a cer fragment inserted between Asc I and Pac I sites. The vector and the cer fragment thus amplified are digested by the Asc I and Pac I enzymes and then ligated together.

- The vector pM1800 thus obtained comprises an expression cassette  
25 under the control of the bacteriophage T7 promoter, a polylinker for cloning the genes of interest downstream from the promoter, a transcription terminator also derived from bacteriophage T7, the cer fragment downstream the polylinker and a kanamycin resistance gene. The DNA sequence of plasmid pM1800 is SEQ ID No 10.

- 30 The XL 1-Blue strain (Stratagene, La Jolla, CA) was transformed with pET8cTat7C/S. Two clones were transplanted and the ADN of the plasmid was extracted and digested with NcoI and HindIII (GIBCO-BRL) restriction enzymes in buffers suggested by the manufacturer. The Tat7C/S DNA sequence (approximately 300 bp) was then isolated on 2%  
35 agarose gel by electroelution.

- 23 -

At the same time, the pM1800 plasmid was also digested by NcoI and Hind III and isolated on 1% agarose gel by electroelution.

The digested Tat fragment and pM1800 plasmid were then subjected to ligation with the T4 ligase (GIBCO-BRL), under the conditions  
5 described by the manufacturer. The ligation product was used to transform the E. coli DH10B strain by electroporation, with the clones being selected in the presence of kanamycin.

The plasmid thus produced containing the Tat7C/S DNA sequence is named pM1815.

10

### Example 3

Fermentation, bacterial cell lysis and protein purification

A seed vial of pM1815 is used to inoculate, a pre-culture of E. coli BL21 ( $\lambda$ DE3) (in Erlenmeyer flask containing the LB2X medium. After 15h to 18h agitation at 37°C, the whole content of the flask is  
15 added to 20 L of GluSKYE4 medium (yeast extract, salts and glucose) in a 30L B. Braun fermenter. When the initial growth phase reaches cell density up to A600 of  $30 \pm 5$ , the synthesis of the Tat protein IIIB 7C/S is induced by the addition of an inducer (IPTG 1mM final). The culture is still maintained for 3 hours under agitation at 37°C  
20 and then the medium is chilled down to 10°C before cell harvesting. The cells are collected by centrifugation and stored at  $< -35^\circ\text{C}$ .



Thawing of bacterial paste (15 g)	•Cellular paste thawed for 1 night at $5 \pm 3^{\circ}\text{C}$
↓	
Suspension and homogenization	•In a buffer of 50 mM Tris-HCl, 0.2M NaCl, benzonase* 5 UI/ml, pH 8.0 in an ice bath at 5 to $10^{\circ}\text{C}$ using an Ultraturax * Addition of benzonase extemporaneously
↓	
Cell lysis	•High-pressure cracking using a Panda microfluidizer at 16000 psi or 1100 bars •Centrifugation at 20000 g at $5 \pm 3^{\circ}\text{C}$ , for 2 hours •Removal of supernatant and its clarification by filtration (0.8/0.2 $\mu\text{m}$ )
↓	
Ammonium sulfate precipitation	•Addition of ammonium sulfate to 1.5 M concentration •Magnetic agitation, for 1 hour at room temperature •1 hour of rest •Centrifugation at 10000g at $20 \pm 3^{\circ}\text{C}$ •Re-suspension of ammonium sulfate (AS) precipitate in a 50 mM Tris- HCl, 8M Urea, 50 mM NaCl, pH8.0 buffer = AS solution. •Filtration through 0.2 $\mu\text{m}$ membrane
↓	
SP Sepharose FF Chromatography volume 20 ml 1.5 cm Flow rate 2 ml/min	•column Equilibrated in a buffer of 50 mM Tris-HCl, 50 mM NaCl, 8M urea •Injection of the filtered AS solution followed by rinsing with pH balance-restoring buffer solution •Removal of the flow through •Elution with increasing ionic strength 50 mM Tris-HCl, 0.3M NaCl, 8M urea, pH 8.0 50 mM Tris-HCl, 0.6M NaCl, 8M urea, pH 8.0 50 mM Tris-HCl, 1.5M NaCl, 8M urea, pH 8.0 •Tat7C/S eluted in the NaCl 0.6 M eluate

The purified Tat protein is stored at -20°C. The buffer of the Tat protein thus purified is preferably replaced with an urea-free buffer such as 50mM Tris-HCl pH 7.5. Furthermore, the Tat protein needs to be sterilized before injection. This step can be easily  
5 done by sterilizing filtration on 0.2  $\mu$ m membrane. The Tat IIIB 7C/S thus isolated is greater than 95% pure, as determined by densitometric analysis on a blue coomassie-stained SDS-PAGE gel. Furthermore, the protein thus purified is substantially exempt of any multimeric forms. Indeed, and contrary to the preparation of the  
10 Tat protein of the prior art, the protein thus produced is a monomeric protein containing less than 1% of multimeric Tat forms. Furthermore, the protein of the invention can be purified at a pH near neutrality without forming aggregates. Furthermore, it appears that the expression level of the protein of the invention is higher  
15 than the expression level of the corresponding wild-type protein. Indeed Wild-type Tat represents 5% of the total soluble proteins whereas Tat7C/S represents at least 15% of the total soluble proteins.

#### Example 4

##### 20 Neutralization and Neutralization Assays

###### Transactivation Assay

The transactivation assay was developed from G.Tosi et al., Eur. J. Immunol. 30, 1120-1126 (2000) and M. Rusnati et al. J. of Biological Chemistry 272, 11313-11320 (1997), allowing the biological activity  
25 of the Tat molecule to be determined in vitro. Stably transfected HeLa-3T1 cells are carrying a plasmid with the LTR sequences of the HIV virus. These LTR sequences function as a promoter for the gene of the chloramphenicol acetyl transferase (CAT) which is a reporter. The addition of Tat to the culture medium causes the synthesis of  
30 CAT, which can be measured with a commercial ELISA test (Boehringer). The results were standardized in relation to the cellular protein concentration.

Figure 3 is showing the transactivating activity of the native Tat, Tat toxoid and Tat7C/S.

##### 35 Neutralization Assay

The incubation of serial dilutions of sera with 40ng/ml of purified native Tat prior to transactivation assay, allows to check for neutralization of transactivation activity by comparison with adequate controls.

- 5 Neutralizing titers are expressed as reverse of the last dilution able to reduce 90% (1log) of the transactivation signal.

The following table shows specific antibody titer and neutralizing titer:

Table 1

Sample tested	Neutralizing titer	Specific antibody titer (log)
Cob #075-5 (Tat Toxoid)	<or = 5	3.79
Cob #075-33 (Tat7C/S)	5	3.45
Cob#074 (native Tat)	5	3.2
Cob#045 (positive control) (hyper-immune anti-Tat serum (CFA))	800	6.3

10

These results clearly indicate that Tat7C/S induce antibodies which neutralize Tat transactivation activity. The neutralizing titer is equivalent to titer obtained with Tat toxoid.

Moreover, this experiment confirm that the neutralization test is  
 15 very sensitive since a neutralizing activity is measured even with low titer sera.

#### Example 5

##### Immunosuppression Assay

The immunosuppressive activity of Tat was measured in vitro by a  
 20 lymphoproliferation assay. Lymphoproliferation was measured by tritiated thymidine incorporation (3H-thymidine) in peripheral blood mononuclear cells (PBMCs) after stimulation by a recall antigen (previously described in Zagury et al., Proc. Natl. Acad. Sci. U S A. 1998; 95:3851-6).

- 25 This assay consisted of isolating, on a ficoll gradient, PBMCs from the peripheral blood of a healthy subject and cultivating them in a microwell in the presence of recall antigen and declining doses of Tat protein in an HL1 culture medium supplemented with  $5 \times 10^{-5}$ M B-mercaptoethanol and 10% AB serum. Each dose of Tat was tested in

triplicate. 18 hours before the cessation of the culture, 0.5 mCi of tritiated thymidine was added to each microwell. The cells were then washed and the incorporated radioactivity was measured with a fluid scintillation counter. The results were measured in cpm.

- 5 The goal of this test was to characterize the immunosuppressive properties of a genetic mutant of Tat. The PBMCs were incubated with 5 µg of native Tat IIIB, detoxified or Tat7C/S), stimulated by the antigens PPD/TT (PPD at 1000 units/ml and TT at 1000 Lf/ml) over a period of 5 days.
- 10 Detoxified Tat is produced by inactivation of Tat IIIB by an alkylation reaction of Tat IIIB (Seq. ID. No.: 1) using iodoacetamide in the following conditions: added micromoles of iodoacetamide = 200 X number of micromoles of Tat + number of micromoles of DTT.
- 15 The results are presented in Figure 1 as % of immunosuppression, calculated as follows:

$$\% \text{ immunosuppression} = \frac{(\text{cpm in cells not treated with Tat}) - (\text{cpm in cells treated with Tat})}{100}$$

- The data represent 3 experiments performed independently on 3 different donors. The results show that under conditions where
- 20 native Tat inhibits the proliferation of PBMCs by 40%, the mutant of Tat7C/S shows no immunosuppressive activity.

#### Example 6

Immunogenicity of the mutant TatIIIB 7C/S in the guinea pig

- Five female guinea pigs (Dunkin-Hartley albinos) were injected two
- 25 times, at two week intervals, intramuscularly (in the quadriceps) with 50 µg of the TatIIIB 7C/S. A control group of five guinea pigs received, in a similar manner, 50 µg of chemically detoxified TatIIIB protein (termed "TatIIIB toxoid" prepared according to the process described in example 5).

- 30 The antibody level induced against the native TatIIIB protein were evaluated by ELISA before and after each immunizations (Days 1, 14, and 29, respectively). The results are displays in figure 2.

- The IgG antibody titers (expressed in log10) are represented in the table 2. The antibody titers of the samples were calculated by
- 35 linear regression of a standard an anti-TatIIIB hyperimmune serum from guinea pig. The titer of this standard serum was first set as

the reciprocal of its dilution, giving an optical density at 450 - 650 nm of 1.0 (average titer calculated at the end of several independent titrations). Limit of detection set at 0.7 log10.

The TatIIIB 7C/S was shown to be capable of inducing specific  
5 antibodies against the native TatIIIB protein in this guinea pig model, with the levels induced after 2 immunizations being very close to those evoked by the TatIIIB toxoid protein.

Table 2

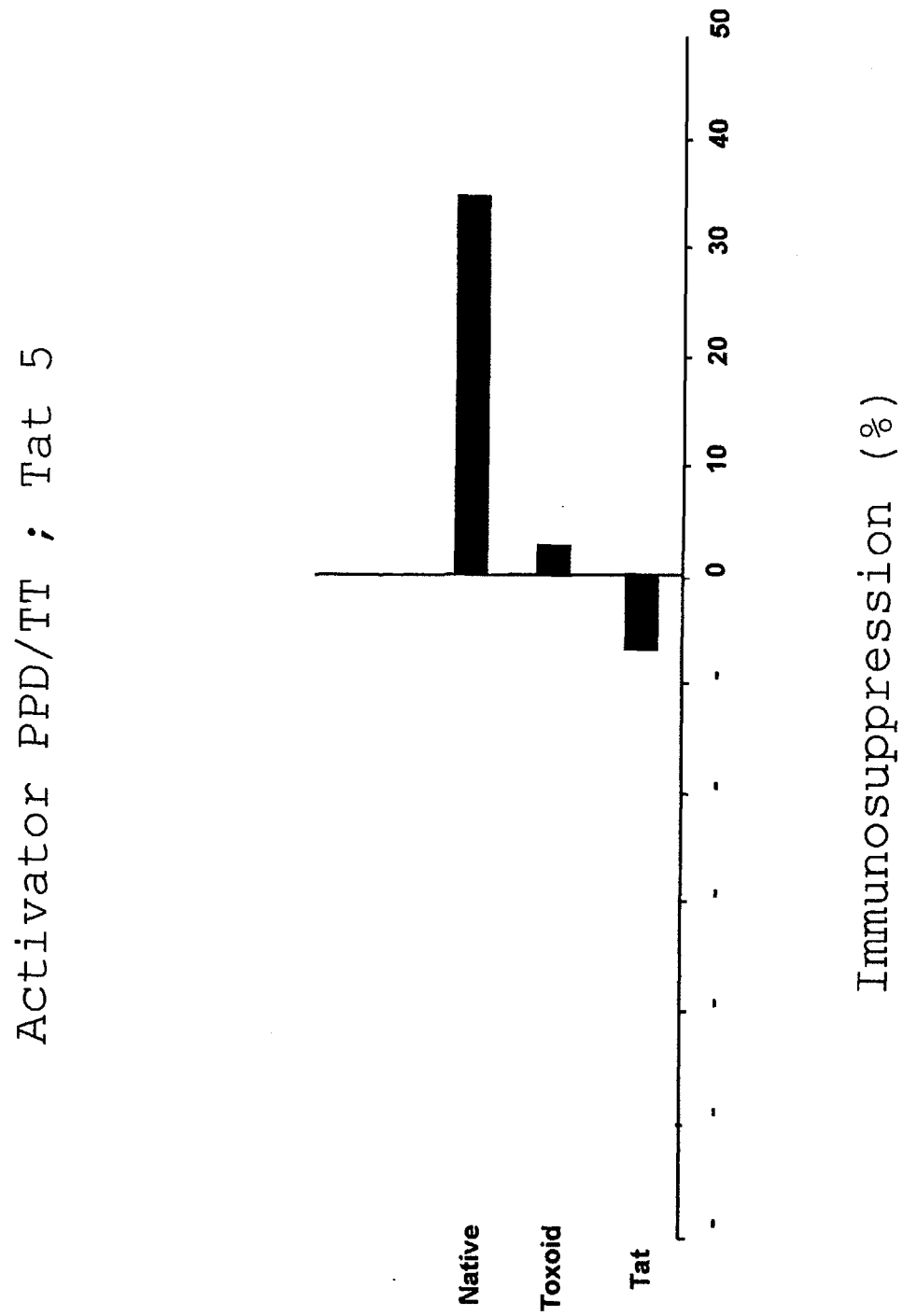
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Immunogen	Guinea pig #	Native anti-TatIIIB IgG antibody titers (log10)		
		Day 1	Day 14	Day 29
TatIIIB 7C/s	1	0	0.000	3.327
	2	0	0.000	3.112
	3	0	1.711	3.458
	4	0	0.000	2.834
	5	0	0.000	3.034
	mean	0	0.342	3.153
	std deviation	0	0.765	0.245
Toxoid TatIIIB	6	0	0.000	3.243
	7	0	1.327	2.967
	8	0	1.522	3.384
	9	0	1.138	Dead
	10	0	2.321	3.796
	mean	0	1.262	3.348
	std deviation	0	0.837	0.346

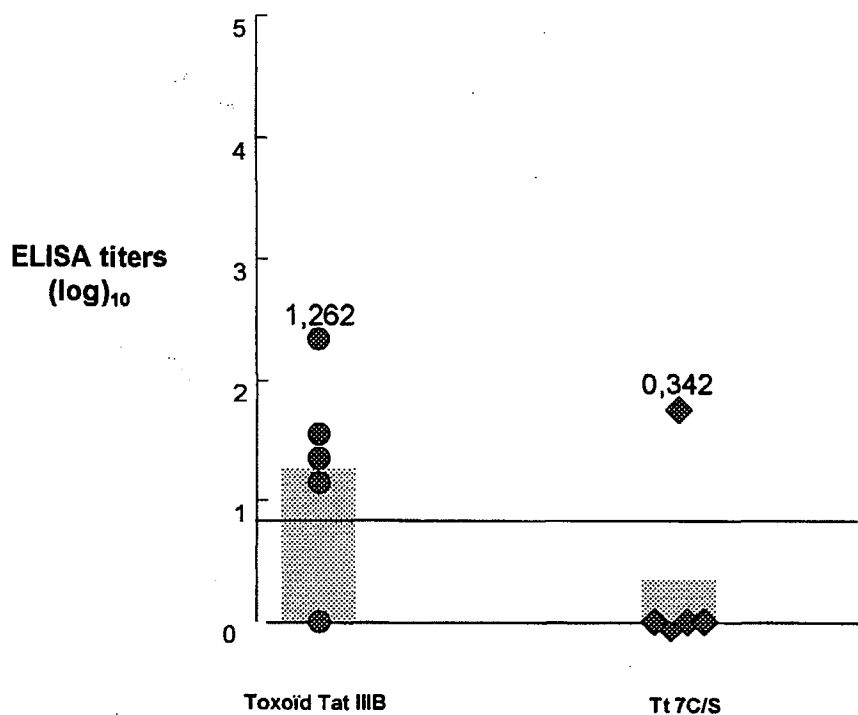
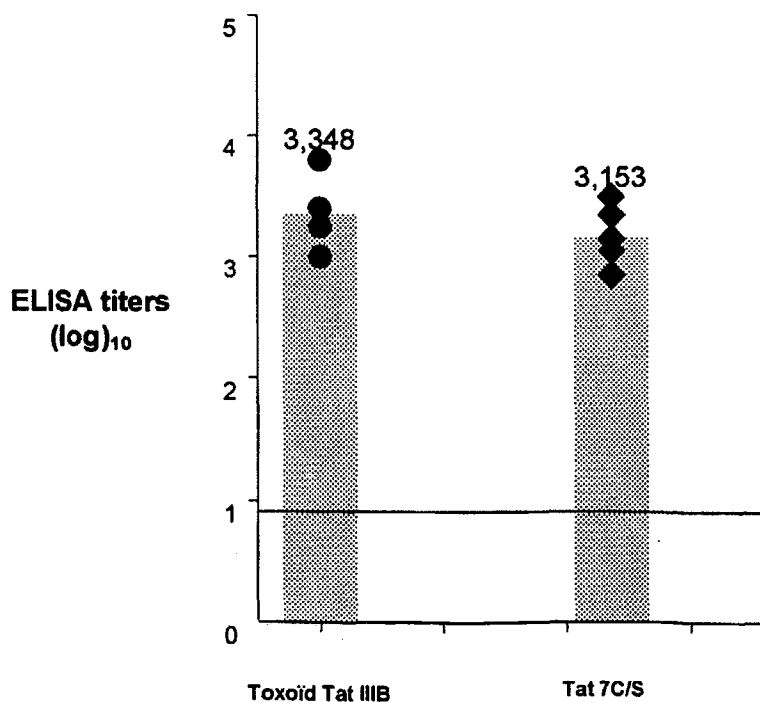
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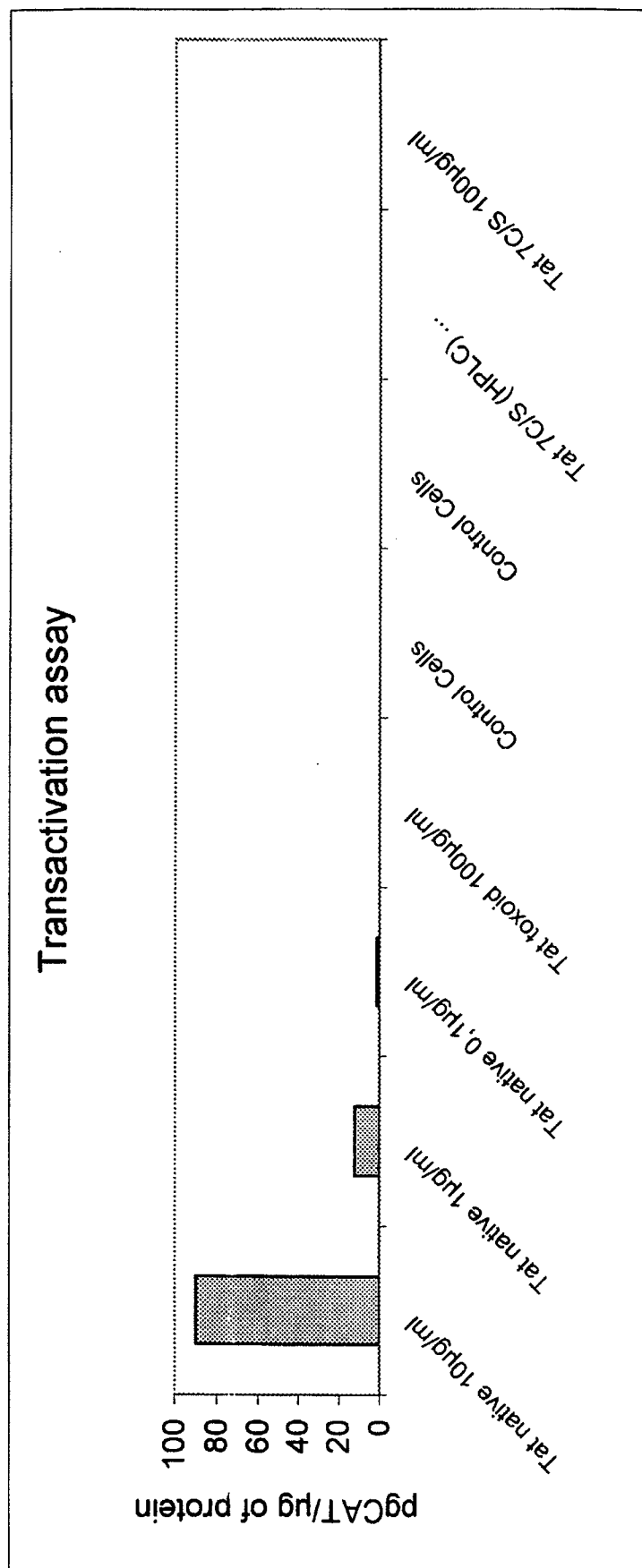
1. A Tat protein comprising a mutated cysteine-rich domain wherein  
5 all the cysteine residues of the cysteine-rich domain have been replaced independently with another amino acid.
2. The Tat protein according to claim 1, wherein each cysteine  
residue of the cysteine-rich domain is a conservative  
10 substitution.
3. The Tat protein according to claim 1, wherein each cysteine  
residue of the cysteine-rich domain is a serine.
- 15 4. A nucleic acid encoding the Tat protein according to any one of claims 1 to 3.
5. An expression vector comprising a nucleic acid according to  
claim 4.  
20
6. The expression vector of claim 5 further comprising a DNA  
sequence encoding Nef and Rev proteins.
7. The expression vector of claim 7 wherein the DNA sequence  
25 encoding the Rev protein is inserted anywhere into the Nef DNA  
sequence encoding amino acids 150-179 of the Nef protein.
8. A composition comprising the Tat protein according to any one of  
claims 1 to 3, carrier and optionally and adjuvant.  
30
9. A composition comprising the expression vector according to any  
one of claims 5 to 7, a carrier and optionally an adjuvant.
10. The composition of claim 8 or 9 comprising at least one Th1  
35 adjuvant.

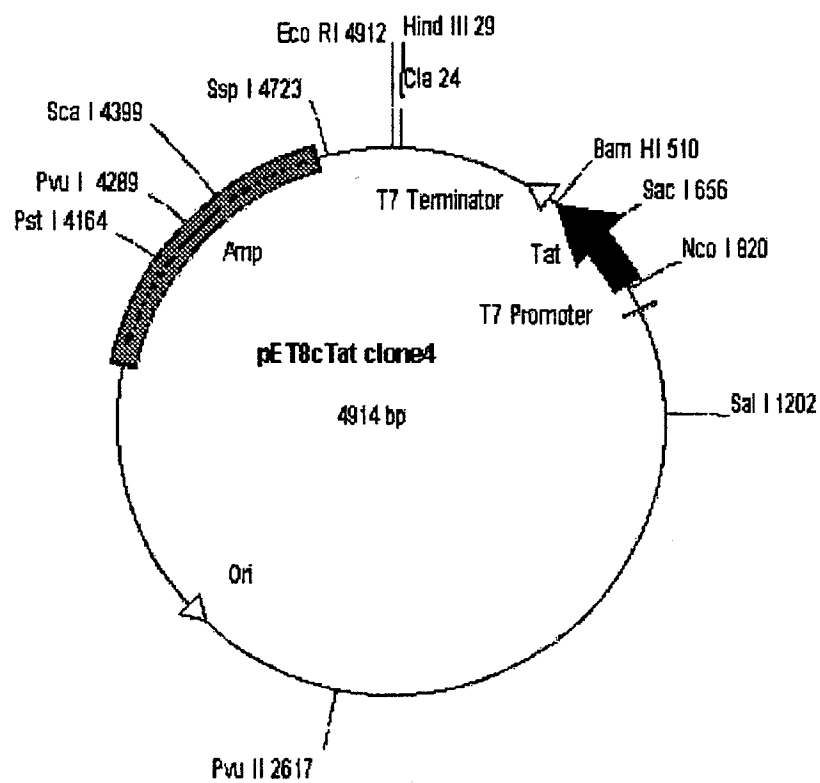
11. A method of eliciting a humoral and cellular immune response in a mammal comprising administering a composition according to any one of claims 8 to 10 to the mammal.
12. The method according to claim 11 wherein the composition of  
5 claim 8 and the composition of claim 9 are administered simultaneously or sequentially.

**Figure 1.**



**Figure 2(a) - Anti-native Tat III B IgG ELISA titers in guinea pigs : D14****Figure 2(b) - Anti-native Tat III B IgG ELISA titers in guinea pigs : D29**

**Figure 3**

**Figure 4**

## SEQUENCE LISTING

<110> Aventis Pasteur S.A.

Rappaport, Jay

Klein, Michel

Zagury, Jean Francois

<120> Mutated HIV TAT

<130> TP019

<160> 11

<170> PatentIn version 3.0

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<211> 86

<212> PRT

<213> Human immunodeficiency virus type 1

<400> 1

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35 40 45

Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr  
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His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp  
65 70 75 80

Pro Thr Gly Pro Lys Glu  
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Organization  
International Bureau



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3 July 2003 (03.07.2003)

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- (72) Inventors; and
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- Published:**  
— with international search report
- (88) Date of publication of the international search report: 4 March 2004
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: MUTATED HIV TAT

(57) Abstract: The present invention provides a Tat protein wherein all the cysteine residues of the cysteine-rich domain have been replaced with another amino acid, preferably with serine, nucleic acids encoding it, and methods of using it to elicit a humoral and cellular immune responses in a mammal. The Tat protein of the invention is therefore useful, inter alia, for prophylactic and/or therapeutic anti-HIV use as well as raising anti-native Tat antibodies in mammals.

WO 2003/054006 A3

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/16 C12N15/49 C12N15/85 A61K39/21 A61K39/39

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, MEDLINE, EMBASE, CHEM ABS Data, SEQUENCE SEARCH, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>GOLDSTEIN G: "HIV - 1 Tat protein as a potential AIDS vaccine"</p> <p>NATURE MEDICINE, NATURE PUBLISHING, CO, US,</p> <p>vol. 2, no. 9, September 1996 (1996-09), pages 960-964, XP002129594</p> <p>ISSN: 1078-8956</p> <p>see in particular Fig. 2</p> <p>the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1-12

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \* & \* document member of the same patent family

Date of the actual completion of the international search

15 July 2003

Date of mailing of the international search report

04/08/2003

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUET T ET AL: "A HIGHLY DEFECTIVE HIV-1 STRAIN ISOLATED FROM A HEALTHY GABONESE INDIVIDUAL PRESENTING AN ATYPICAL WESTERN BLOT" AIDS, LONDON, GB, vol. 3, no. 11, November 1989 (1989-11), pages 707-715, XP000867752 ISSN: 0269-9370	1-5
Y	page 712, right-hand column, paragraph 1 -page 713, left-hand column, paragraph 1	6-12
X	HOFFMANN S ET AL: "A SELECTION SYSTEM TO STUDY PROTEIN-RNA INTERACTIONS: FUNCTIONAL DISPLAY OF HIV-1 TAT PROTEIN ON FILAMENTOUS BACTERIOPHAGE M13" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US, vol. 235, 1997, pages 806-811, XP002918780 ISSN: 0006-291X	1-5
Y	the whole document	6-12
X	CASELLI E ET AL: "DNA immunization with HIV - 1 tat mutated in the trans activation domain induces humoral and cellular immune responses against wild-type Tat" JOURNAL OF IMMUNOLOGY, THE WILLIAMS AND WILKINS CO. BALTIMORE, US, vol. 162, no. 9, 1 May 1999 (1999-05-01), pages 5631-5638, XP002129597 ISSN: 0022-1767	1-5
Y	the whole document	6-12
X	SADAIE M R ET AL: "CONSERVATIVE MUTATIONS IN THE PUTATIVE METAL-BINDING REGION OF HUMAN IMMUNODEFICIENCY VIRUS TAT DISRUPT VIRUS REPLICATION" AIDS RESEARCH AND HUMAN RETROVIRUSES, vol. 6, no. 11, 1990, pages 1257-1264, XP009013912 ISSN: 0889-2229	1-5
Y	the whole document	6-12
X	MITOLA STEFANIA ET AL: "Identification of specific molecular structures of human immunodeficiency virus type 1 Tat relevant for its biological effects on vascular endothelial cells." JOURNAL OF VIROLOGY, vol. 74, no. 1, January 2000 (2000-01), pages 344-353, XP002247692 ISSN: 0022-538X	1-5
Y	the whole document	6-12

-/--

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CAPUTO A ET AL: "Inhibition of HIV-1 replication and reactivation from latency by tat transdominant negative mutants in the cysteine rich region." GENE THERAPY, vol. 3, no. 3, 1996, pages 235-245, XP009013907 ISSN: 0969-7128	1-5
Y	the whole document ----	6-12
X	WO 99 27958 A (ENSOLI BARBARA ;IST SUPERIORE SANITA (IT)) 10 June 1999 (1999-06-10)	1-5
Y	the whole document ----	6-12
A	BUANEC LE H ET AL: "A PROPHYLACTIC AND THERAPEUTIC AIDS VACCINE CONTAINING AS A COMPONENT THE INNOCUOUS TAT TOXOID" BIOMEDICINE AND PHARMACOTHERAPY, ELSEVIER, PARIS, FR, vol. 52, no. 10, 1998, pages 431-435, XP000867754 ISSN: 0753-3322 the whole document -----	

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP 02/14841

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 11 and 12 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9927958	A	10-06-1999	IT RM970743 A1	01-06-1999
			AU 2412699 A	16-06-1999
			BR 9814725 A	03-10-2000
			CA 2311647 A1	10-06-1999
			CN 1283121 T	07-02-2001
			WO 9927958 A2	10-06-1999
			EP 1035865 A2	20-09-2000
			JP 2001524531 T	04-12-2001
			PL 341818 A1	07-05-2001
			TR 200001553 T2	21-11-2000

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